1. **Chemical Name(s):** Thiamethoxam, Clothianidin

2. **PC Code:** 060109, 044309

3. **CAS No:** 153719-23-4, 210880-92-5

4. **MRID:** None

5. **ECOTOX Record Number and Citation:** Cutler GC and Scott-Dupree CD. 2014. A field study examining the effects of exposure to neonicotinoid seed-treated corn on commercial bumble bee colonies. Ecotoxicology 23(9):1755-63

6. **Purpose of Review:** Clothianidin and thiamethoxam re-evaluation for pollinators.

7. **Date of Review:**

8. **Description of Use in Document:** Supplemental--Qualitative

9. **Summary of Study Findings:**

Executive Summary

The study looked at bumble bee colony responses when placed adjacent to clothianidin and/or thiamethoxam seed-treated (conventional fields) or organic corn fields for 5-6 days. Corn pollen represented a small component of bumble bee forage (<0.1%—1.8% in conventional fields, <0.1%--2.6% in organic fields). Residues of thiamethoxam were below the level of detection (LOD) of 0.1 ng/g in pollen collected from all fields, whether organic or conventional. Residues of clothianidin were also below the LOD in pollen collected from organic fields, and were between the LOD and the LOQ (0.5 ng/g) in pollen collected from three of the four conventional fields and measured 0.8 ng/g in pollen collected from the final conventional field. Biological parameters measured for the colonies included colony weight, number of honey pots, number of pollen pots, number of brood cells, number of workers, worker weight, number of drones, drone weight, number of queens and queen weight. Statistical differences between colonies adjacent to conventional fields and organic fields were detected only for the number of workers (25% inhibition, 96 ± 15 workers per hive at the conventional fields compared to 127 ± 17 workers per hive at the organic fields), though other parameters (*i.e.* worker weight and drone weight) also were reported to have high, though non-significant differences (>25% for both parameters). Surveys of insects on tassels of dehiscing corn in the two areas generally did not show significant differences, though solitary bees (*i.e. Andrenidae, Halictidae*) were found on dehiscing tassels at

conventional fields almost three times more frequently than on corn tassels in organic fields. Though not statistically significantly different, honey bees (*Apis mellifera*) were observed on dehiscing corn tassels on organic fields more than 7 times more frequently than on corn tassels in conventional fields.

Methods

8 corn fields (4 conventional (2 fields near Elora and 2 near Guelph, Ontario), and 4 organic (2 fields near Teviotdale and 2 near St. Mary's, Ontario)) within 100 km of each other were used in the experiment. There was a minimum distance of 9 km between all fields. Conventional fields were 8—44 hectares large while organic fields were 6—18 hectares. At organic fields, Pioneer P9675 corn seed containing no pesticide (or genetic modification) was grown. At the conventional fields, planted corn seed was modified for expression of Bt (*Bacillus thuringeiensis*) endotoxin and were treated with either thiamethoxam and/or clothianidin. At the conventional sites, various fungicides were used in the seed treatments including ipconazole, metalaxyl (and metalaxyl-M), trifloxystrobin, fludioxinil, azoxystrobin, mefanoxam, thiabendazole. At conventional site 1 (CV1) an additional foliar application of the fungicide pyraclostrobin was made while bumble bees were concurrently in the field. At CV1 and CV2, the neonicotinoid seed treatment used was Poncho 250, containing clothianidin applied at 0.25 mg a.i./seed. At CV3 and CV4, a combination of seeds were planted including seed treated with either Poncho 250 or Cruiser 5FS (containing 0.25 mg a.i. thiamethoxam/seed).

Commercial bumble bee (*Bombus impatiens*) colonies, obtained from Biobest (Leamington, Ontario) were placed adjacent to each control and treatment field. These multi-hive colonies consisted of three colonies (22 x 27 x 15 cm) housed in a weather resistant sytrofoam box and provided with Biogluc® as a carbohydrate source. Each unit was provided with Biogluc (Biobest, Leamington, ON) as a carbohydrate source, but no pollen supplement was provided. Colonies were placed adjacent to the fields (Day 0) when 25—35% of anthers were observed to be dehiscing and silks visible on over 50% of 75 randomly selected corn plants. Colonies remained in the fields for 5 or 6 days of exposure during corn pollen shed. As plantings included different corn hybrid seeds and at slightly different times, the completion of pollen shed varied and ranged from July 27 to August 9 across all sites. At nighttime on Day 5 or 6, colony entrances were shut and the colonies transported to a site near Meaford, ON, that was approximately 165 km northeast of Guelph, and also that authors reported to be isolated from any crops grown from seeds treated with neonicotinoids by approximately 10 km. At this site, the colonies were able to forage on a variety of wildflowers for 30—35 days before being placed in a freezer at the University of Guelph for colony biological assessments. Colony assessments were conducted on an individual colony basis (24 total colonies). Parameters measured included worker, drone and queen weight (total dry weights) as well as total colony weight. Number of honey pots, pollen pots and brood cells (i.e. cells containing eggs, larvae, or pupae) was then recorded.

Foraging activity in the corn fields in areas near the bumble bee colonies was determined on Days 1-4. On each day, between the hours of 12:50 pm and 1:15 pm, observations of pollinator and other insect activity on nearby tassels (≤ 2m away from observation station) were made in four different locations over a 5 minute sampling period (*i.e.*. 20 minutes of observation/site/day). An observation was considered landing and sustained activity/movement of at least 2 seconds on corn tassels. When all hives were at the Meaford site on Aug 27, the number of *B. impatiens* foragers entering and exiting each hive (3 per multi-hive) was recorded over a 5 minute period.

Pollen was collected from bees returning with pollen loads to each multi-hive (n=18) during the pollen dehiscence period at each hive. Bees were captured individually and placed in a -20^0 freezer. Pollen loads (mg) per bee were measured and pollen from each site was then pooled, suspended in a mixture with 25:1 distilled water, mixed and homogenized. Slides of the pollen were then prepared using a subsample of one small drop of the pollen preparation. 500 pollen grains per slide were then identified to species, genus, family or pollen type.

To determine residues of clothianidin and thiamethoxam in pollen, a minimum of 15 randomly selected dehiscing tassels were taken from corn plants within a 50 m^2 area near hives at each site. Tassels were then taken to the laboratory and shaken so that pollen would fall on paper until either all pollen was released or 3 grams of pollen had been collected. Tassels from different sites were placed in different rooms to reduce the possibility of cross-contamination of samples. Pollen was sieved to remove debris prior to residue analysis. Analyses were performed using liquid chromotragraphy-tandem mass spectrometry (LC-MS/MS) with a limit of detection (LOD) of 0.1 ng/g and a limit of quantification (LOQ) of 0.5 ng/g.

Statistics

Analyses were performed using JMP 10.0 software (SAS Institute, 2012). The study authors considered the replicate unit to be the field site and for colony measurements, data from the three colonies in each multi-hive were averaged to determine the replicate average. There were inadequate degrees of freedom to do a multivariate analysis of variance, so t-tests were conducted on hive endpoints to compare effects of exposure to conventional corn grown from neonicotinoid treated seed, to effects of exposure to organic corn grown from untreated seed. Data met the assumptions of normality (Shaprio-Wilks test) and homogeneous variance (O'Brien test). Pollinator and other insect foraging activity in the test sites was analyzed using a Wilcoxon test (20 minutes of observation/site with day as a blocking factor) as was post-exposure hive entry and exit counts (hive as a blocking factor) since residuals and variance assumptions were not met for these data.

Results

Residues of thiamethoxam were below LOD of 0.1 ng/g in pollen collected from dehiscing corn tassels in all fields, whether organic or conventional. Residues of clothianidin were also below

the LOD in pollen collected from organic fields, and were between the LOD and the LOQ (*i.e.* between 0.1 and 0.5 ng/g) in pollen collected from three of the four conventional fields and measured 0.8 ng/g in pollen collected from the final conventional field. See table 3 for further details.

Corn pollen was a small component of bumble bee forage, measuring <0.1%—1.8% in pollen collected from bumble bee foragers in conventional fields, and <0.1%-2.6% in pollen collected from bumble bee foragers in organic fields. Major sources of bumble bee collected pollen were *Solanum dulcamara* (23%-96% of bumble bee forage, >66% in five sites), *Lotus sp.* (31—43% of bumble bee forage in 3 sites, <10% in the remaining sites), and Type *Taraxacum* (may have included *Taraxacum, Arnoseris, Chichorium, Crepis, Hieracium, Hypochoeris, Lactuca, Laspsana, Leontodon, Picris, Sonchus,* and *Tragopogon*; 12%-26% in the organic sites, <10% in the conventional sites). See Table 4 for further details.

Surveys of insects on tassels of dehiscing corn in the two areas generally did not show significant differences, though solitary bees (*i.e. Andrenidae, Halictidae* families) were found on dehiscing tassels at conventional fields almost three times more frequently than on corn tassels in organic fields ($p = 0.007$). Though not statistically significantly different, honey bees (*Apis mellifera*) were observed on dehiscing corn tassels on organic fields more than 7 times more frequently than on corn tassels in conventional fields ($p = 0.22$), while *B. impatiens* were observed on dehiscing corn tassels in conventional fields more than 6 times more frequently than on corn tassels in organic fields ($p = 0.27$). During the post-exposure period when colonies were in Meaford, ON (August 27), there was reported to be no statistical difference between the number of entries and exits in the colonies that were previously near conventional fields (9.4 ± 4.0 entries and exits) and those that had been near organic fields (11.7 ± 10.3 entries and exits). See Table 1 and 2 for further details.

Table 1 (reproduced from Cutler and Dupree, 2014) summarizes the results for the bumble bee colony biological assessment endpoints. Statistically significant differences between colonies placed near conventional fields and colonies placed near organic fields were not observed for any endpoint, except for number of workers (24.9% inhibition, $p = 0.032$). Worker weight (26.4% inhibition, $p = 0.078$) and drone weight (34.2% inhibition, $p = 0.10$) were also observed to have high, though not statistically significant differences in bumble bees from colonies placed near organic fields than in colonies placed near conventional fields.

Table 1 Effects (mean ± SD) on commercial Bombus impatiens colonies when exposed during pollen shed to corn (Zea mays) grown from conventional seed treated with neonicotinoid insecticide or certified organic seed, Ontario 2013

Endpoint measure (per hive)	Corn seed type		t test statistics
	Conventional	Organic	
Hive weight (g)	883.3 (156.2)	843.2 (80.4)	$t_6 = -0.46$, P = 0.66
No. honey pots	331.3 (127.8)	270.2 (55.7)	$t_6 = -0.88$, P = 0.41

No. pollen pots	32.6 (21.9)	19.2 (5.0)	$t_6 = -1.19$, P = 0.28
No. brood cells	554.8 (93.9)	505.0 (54.7)	$t_6 = -0.91$, P = 0.39
No. workers	96.0 (15.1)	127.9 (17.2)	$t_6 = 2.80$, P = 0.032
Worker weight (g)[a]	6.7 (1.9)	9.1 (1.2)	$t_6 = 2.12$, P = 0.078
No. drones	99.5 (41.0)	112.1 (10.6)	$t_6 = 0.59$, P = 0.57
Drone weight (g)[a]	7.3 (3.4)	11.1 (1.9)	$t_6 = 1.90$, P = 0.10
No. queens	9.2 (2.1)	7.5 (1.2)	$t_6 = -1.41$, P = 0.21
Queen weight (g)[a]	3.1 (0.9)	2.2 (0.4)	$t_6 = -1.82$, P = 0.12

[a] Total dry weight of all bees

Table 2 (from Cutler and Dupree, 2014) summarizes the results for the number of insects on tassels of dehiscing corn grown from conventional and treated seed. No significant differences were observed.

Table 2 Number of insects (mean ± SD) on tassels of dehiscing corn (*Zea mays*) grown from conventional seed treated with neonicotinoid insecticide or certified organic seed, Ontario 2013

Insect	Number insects counted (20 min)		Wilcoxon statistics
	Conventional	Organic	
Bombus impatiens	0.37 (0.76)	0.06 (0.36)	$Z = -1.10$, $P = 0.27$
Other *Bombus*	0.31 (0.54)	0.13 (0.37)	$Z = -1.05$, $P = 0.29$
Apis mellifera	0.06 (0.27)	0.44 (0.82)	$Z = 1.23$, $P = 0.22$
Solitary bees	4.25 (3.61)	1.44 (1.63)	$Z = -2.70$, $P = 0.007$
Other insects	9.00 (4.62)	8.50 (4.18)	$Z = 0.13$, $P = 0.89$

Table 3 (from Cutler and Dupree, 2014) summarizes the results for the residue analysis.

Table 3 Residues of thiamethoxam or clothianidin in pollen collected from corn (*Zea mays*) plants grown from conventional seed treated with neonicotinoid insecticide or certified organic seed, Ontario 2013

Field	Clothianidin (ng/g)	Thiamethoxam (ng/g)
Conventional 1	0.8	<0.1[a]
Conventional 2	0.4	<0.1
Conventional 3	0.1	<0.1
Conventional 4	0.3	<0.1
Organic 1	<0.1	<0.1
Organic 2	<0.1	<0.1
Organic 3	<0.1	<0.1
Organic 4	<0.1	<0.1

[a] Limit of detection = 0.1 ng/g

Table 3 (from Cutler and Dupree, 2014) Analysis of pollen types recovered from worker bees returning to hives found that a very low portion of corn pollen was collected. Pollen samples recovered from bees at most sites were dominated by *Solanum dulcamara* (bittersweet nightshade), although 10–40 % of some samples consisted of Type *Taraxacum*, *Lotus* (e.g. bird's-foot trefoil, deervetches), Type *Trifolium hybridum*, or *Coronilla*. Depending on the site, pollen from 4 to 12 other floral resources was found in lower amounts.

Table 4 Floral sources (species, genus, or family) used by commercial *Bombus impatiens* colonies when exposed during pollen shed to corn (*Zea mays*) grown from conventional seed treated with neonicotinoid insecticide or certified organic seed, Ontario 2013

Field	Pollen type[a]	Percentage total pollen
Conventional 1	*Lotus*	42.6
	cf. *Solanum dulcamara*	35.2
	Coronilla	9.6
	Z. mays	0.8
	Other (10)	11.8
Conventional 2	cf. *S. dulcamara*	38.4
	Lotus	34.0
	Type *Trifolium hybridum*[b]	11.2
	Z. mays	1.8
	Other (8)	14.6
Conventional 3	cf. *S. dulcamara*	96.4
	cf. *Hypericum*	1.2
	Type *Taraxacum*[c]	1.0
	Z. mays	0.0
	Other (4)	1.4
Conventional 4	cf. *S. dulcamara*	89.0
	cf. *Medicago sativa*	6.0
	Type *T. hybridum*	1.8
	Z. mays	0.0
	Other (7)	3.2
Organic 1	*Lotus*	31.4
	cf. *S. dulcamara*	23.4
	Type *Taraxacum*	12.4
	Z. mays	0.0
	Other (12)	32.8
Organic 2	cf. *S. dulcamara*	70.8
	Type *Taraxacum*	11.8
	Arctium	7.6
	Z. mays	0.0
	Other (4)	9.8
Organic 3	cf. *S. dulcamara*	67.6
	Type *Taraxacum*	25.6
	Cirsium or *Carduus*	2.2
	Z. mays	0.0
	Other (4)	4.6
Organic 4	cf. *S. dulcamara*	67.2
	Type *Taraxacum*	23.2
	Z. mays	2.6
	cf. *M. sativa*	2.2
	Other (6)	4.8

[a] For brevity, only the top-three floral sources and portion of corn pollen detected in pollen samples are listed. Values in parentheses indicate the number of other pollen types found

[b] May include (share the same palynological features) *T. hybridum*, *T. agrarium*, *T. arvense*, *T. repens*, and *Medicago lupulina*

Data Quality Evaluation

No standard performance criteria for bumble bee colonies in control conditions have currently been identified by EPA or PMRA; therefore the performance exhibited by control colonies in this experiment cannot be assessed. Similarly, no existing guidelines are available for field tests conducted with *Bombus impatiens*. Replication in the experiment was low (n = 4 sites, with only one multi-colony per site), which makes the ability to statistically distinguish differences between

control and treatment groups difficult, especially for field tests on colony-level parameters, which traditionally have high variability. With this level of replication and with only one treatment dose, it is unknown if the effects that were statistically observed in the treatment group (number of workers) is a random deviation or truly significant. Similarly, it is uncertain whether the high (>25% inhibition), but statistically insignificant differences observed in the treatment group (worker weight and drone weight) are true biological effects or are within the range of normal variability. An additional potential uncertainty exists in that the fields in the treatment group were exposed to different neonicotinoid treatments (CV1 and CV2 were only treated with clothianidin, while CV3 and CV4 had a mixture of seeds treated with either clothianidin or thiamethoxam). The study authors did not report how hives were initially assigned to control and treatment fields. Generally, in field tests with pollinators, a recommended methodology would use a randomized design blocking for colony strength by initial colony weights.

The residue tests on corn pollen directly collected from dehiscing corn tassels did have adequate replication (n = 15), however comparisons between the conventional fields is complicated since these fields' treatments were slightly differently. The LOD and LOQ determined in this study are within the precision typically expected in residue analysis for neonicotinoid compounds.

Peer Review

Primary Reviewer Comments

Rationale for Use:
This study provides information on residues of clothianidin and thiamethoxam following seed treatment on corn. Most regulatory testing is performed on honey bees (*Apis mellifera*), so this study provides information that contributes to the understanding of potential thiamethoxam and clothianidin exposure to bumble bee colonies from corn pollen following seed treatment. Exposures are sub-lethal and may represent environmentally relevant scenarios from typical corn use patterns (though not from maximum labeled use patterns).

Limitations of Study:
The seed treatments used (all 0.25 mg a.i./seed, either thiamethoxam or clothianidin) are well below the maximum rates labeled for use on corn seed in the United States and Canada (both labeled at 5x the tested rate, for a maximum rate of 1.25 mg a.i./seed). Since residues were unable to be quantified (*i.e.* below the LOQ) in three of the four treatment fields, this information cannot be scaled up to reflect the higher labeled maximum application rate. However, qualitatively, from this data it seems likely that applications at the maximum rate on corn seed will likely result in residues in corn pollen below 10 ppb, which agrees with other available residue data for treated corn seed.

However, extrapolations of the effects data from this study cannot be made since the application rate in the study was well below the maximum labeled rate. Furthermore, the low sample size results in a lack of confidence around the effects endpoints. It is unclear whether the only

statistically significant biological effect seen (inhibition of the number of workers produced) is truly significant, or a random deviation. Similarly, it is unclear whether relatively high, but not statistically significant, inhibitions of worker and drone weight are within expected variation for these parameters or potentially masked by the small sample size of the study. Foraging activity was evaluated for four days. The exposure period was 5-6 days during corn pollen shed. It was unclear from the study authors' methods why some colonies were exposed for five days and others six or what criteria the authors used to determine removal of the colonies. Corn pollen shed in a field can continue for up to 14 days (Nielsen, 2010). Therefore, the exposure that the study authors tested may not be representative of potential actual conditions to which bumble bee colonies may be exposed.

Although pollen was collected from bumble bees and identified to various taxa levels, no residue analysis was conducted on the bee-collected pollen which may have been useful to identify whether any additional confounding effects may have occurred through contamination of control colonies (from residues in pollen and nectar of other floral sources brought into the hive). The study authors reported that crops grown in the surrounding area of the post-exposure location in Meaford, ON were not grown from neonicotinoid treated seeds. However, the study authors do not report on whether there was potential that foliar or soil applied neonicotinoid treatments may have been applied to crops in the surrounding area of the post-exposure location. Residue analysis of hive-stored pollen from both the exposure and post-exposure periods would have helped reduced this additional source of uncertainty.

Some conventional sites were not tested for residues of both clothianidin and thiamethoxam. They were only tested for one active ingredient which posed a problem for the sites where a combination of neonicotinoid seed treatments were planted:

- "We did not detect thiamethoxam in pollen samples from conventional sites CV3 and CV4, which were planted with seeds treated with both clothianidin and thiamethoxam. It is probable that the tassels we collected in the field were from those plants treated with clothianidin rather than thiamethoxam. It is also possible that thiamethoxam on treated seed was metabolized to clothianidin, as may occur with foliar sprays or irrigation treatments of thiamethoxam or on corn seeds treated with thiamethoxam."

Treatments were not consistent between conventional sites (i.e. one site had seed treated with Poncho 250, another site had seed treated with Cruiser 5FS). This occurred because the authors worked with independent growers to conduct the trial under real world field conditions. Conventional fields were all consistent in that corn was grown from seeds that express Bt Cry toxins, and were treated with neonicotinoids and fungicides, but specific pesticide treatments used were not identical. Our study therefore might be considered more along the lines of a monitoring study or ''quasi-experiment''. The authors' admit that this could lead to uncertainty about conclusions.

Development of corn plants at organic sites was slower than that at conventional sites. Hives were therefore placed in organic fields approximately a week later than in conventional corn fields. This staggered placement of hives in conventional vs. organic fields might have resulted in hives that differed in worker production.

It rained (~9.6–19.5 mm) on both evenings when bees were collected (21:00–22:00) from conventional sites, whereas it rained (~18.6 mm) only one of two evenings during collections at organic fields (Anonymous 2014). Given that bumble bees may seek shelter under foliage if it is raining and may not immediately return to their nest (Benton 2006), the additional rain at conventional sites may have meant fewer foraging workers had returned to their hives when they were collected.

Foraging activity in the corn fields in areas near the bumble bee colonies was determined on days 1 to 4. It is unclear why the study author did not include days 5 to 6, at which there may have been more time for the toxin to build up from dietary exposure to cause forager disorientation.

Description of Use in Document (QUAL, QUAN, INV):
Qualitative. The effects data presented here should not be used quantitatively in risk assessment (due to the limitations reported here, including lack of adequate replication and lower than maximum labeled application rates). The residue data may be used qualitatively for risk assessment purposes. Additionally, the pollen analysis provides useful information to characterize the utility of corn pollen in bumble bee colonies.

Primary Reviewer: Michael Wagman, EPA/EFED/ERB6

MICHAEL WAGMAN

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Secondary Reviewer: Barbara Martinovic Barrett, EAD

Secondary Reviewer Comments:

This study provides effects data for a non-*Apis* species (*Bombus impatiens*) and residue data on pollen from treated corn seed. Even though this study has limitations (see above), results suggest that exposure to corn grown from neonicotinoid-treated seed during pollen shed poses a low risk to *B. impatiens*. This study also showed that very little corn pollen was actually collected by the bumble bees despite the hives being located directly next to the corn field during pollen shed. The only statistically significant effects reported in the study were that: (1) more solitary bees were observed on tassels in conventional than organic fields; and (2) fewer workers were recovered from hives placed next to conventional fields. This study is therefore considered to provide useful information that can enhance the understanding of intra-species variability when assessing the risk to native bees under real world field conditions.

Resolution:

[Provide a description of the resolution if there is a discrepancy between the primary and the secondary reviewer]

10. References:

Cutler, G.C. and C.D. Scott-Dupree. 2014. A field study examining the effects of exposure to neonicotinoid seed-treated corn on commercial bumble bee colonies. Ecotoxicology. 23(9):1755-63

Nielsen, R.L. 2010. Tassel Emergence & Pollen Shed. Corny News Network. Purdue University. [On-line]. Available at http://www.agry.purdue.edu/ext/corn/news/timeless/tassels.html [URL accessed January, 2015]